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Studies on β-sitosterol and ceramide-induced alterations in the properties of cholesterol/sphingomyelin/ganglioside monolayers

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ABSTRACT

Phytosterol—β-sitosterol promotes apoptosis in various cancer cells and inhibits their growth. Supplementation of cancer cells with this compound causes modifications in membrane composition, namely, substitution of cholesterol (Chol), decrease of sphingomyelin (SM) content and increase of ceramide (Cer) level. The aim of this work was to investigate the influence of partial replacement of cholesterol by plant sterol, substitution of sphingomyelin by ceramide and both these factors simultaneously on the properties of the monolayers composed of major lipids identified in breast cancer membranes, namely Chol/SM/GM3 mixtures. Brewster Angle Microcopy experiments and the analysis of the isotherms recorded during films compression and resulting parameters evidenced that β-sitosterol weakens the interactions between molecules, decreases films stability and condensation. The influence of ceramide on sterol/SM/GM3 films was reflected in strong modifications of their texture, however, the morphology of monolayer was determined by the structure of sterol present in the system. It was also found, that simultaneous replacement of 50 mol% of Chol and SM by phytosterol and Cer, respectively, induces lipids segregation, which is manifested in large diversity of phases observed in BAM images. To facilitate the analysis of the data collected for multicomponent monolayers, the properties of selected sterol/GM3, sterol/Cer, SM/GM3, Cer/GM3 binary films were also investigated. The obtained results evidenced that the studied herein modifications in the composition of Chol/SM/GM3 monolayer, reflecting compositional alterations induced by phytosterol in cancer membranes, strongly affect the organization of model system, therefore they should be considered in the studies on anticancer mechanism of β-sitosterol.

1. Introduction

It is known that the components of membranes are distributed heterogeneously and within the membrane microdomains (e.g. rafts) of various compositions are formed. These assemblies are essential for functioning of membranes and cells [1,2] and the disturbances in their organization are frequently connected with serious diseases [3]. Domains play also critical role in development, growth, progression and metastasis of cancer cells [4–6] and they are considered as a target in cancer therapy [4,7,8]. Therefore, a lot of attention is paid to the issue of disordering in functionality of cancer cells induced by the depletion of cholesterol in membrane and rafts [7]. In these studies cholesterol is usually eliminated by using methyl-β-cyclodextrin, polyene antibiotics or statins [5,7,9,10]. It was reported that depletion of cholesterol in membrane reduces formation of rafts/caveolae and decreases their stability as well as affects signaling processes, cell proliferation and viability [8,10]. The studies confirmed also that depletion of cholesterol changes morphology and promotes apoptosis leading to death of various cancer cells [7,8,11,12]. Interestingly, re-addition of cholesterol provokes reappearance of rafts/caveolae and reverses the effects of cholesterol depletion [7,8,11]. Moreover, cancer cells, probably due to higher level of cholesterol and larger number of rafts, are more susceptible to the effects of sterol depletion than normal cells [8]. All these results prove that the content of cholesterol in membrane is of vital importance to cancer cell viability, thus it can be assumed that the compounds capable of modifying cholesterol concentration in cancer membranes may be considered as potentially helpful in cancer treatment. A good example of compound, which is able to incorporate into animal membranes and manipulate sterols level is the plant sterol — β-sitosterol [13–18]. The studies on human leukemic cells (U937) [19], colon (HT-29, COLO 320 DM, HT116) [20–24], prostate (LNCaP, PC-3) [24–26], breast (MDA-MB-231, MCF-7) [27–30] or stomach (SGC-7901) [31] tumor cells evidenced, that supplementation with β-sitosterol inhibits the growth and promotes apoptosis in various cancer cells. It was found that treatment of cancer cells with phytosterol results in activation of caspase-3, endoreduplication and G2/M arrest, DNA damage and expression of *Fas* death receptor as well as affects activation of membrane bound enzymes [19,23,24,30,32]. The studies on stomach cancer cell proved also that β-sitosterol disorganizes the cells and changes their density making them more elongated [31]. The consequences of β-sitosterol supplementation appear also at the level of membrane

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and they cover modifications in membrane properties [31,24] and composition [20,28]. For example, treatment of colon cancer cells with β-sitosterol decreases membrane cholesterol and sphingomyelin level [20]. Reduction of cholesterol by β-sitosterol without decrease of total sterols level was also found for breast cancer cells [28] and it was suggested that phytosterol may replace cholesterol in membrane [33]. Moreover, supplementation of colon, prostate and breast cancer cells with β-sitosterol increases ceramide level [21,26,29,33]. All these modifications are essential from the point of view of organization of membranes and rafts. It is known that ceramides are able to self associate and form microdomains, which then spontaneously fuse into large platforms [34,35]. It was also reported that ceramides compete with cholesterol, which is raft-stabilizer, and they are able to replace sterol in domains [36]. Thus, the variations made by β-sitosterol in cancer membrane composition may alter lipid distribution in membrane and rafts and their properties.

In this work Langmuir monolayers composed of major raft components identified in breast cancer membranes, that is cholesterol (Chol)/sphingomyelin (SM)/ganglioside (GM3) monolayers [37] were investigated in order to verify how the replacement of cholesterol by plant sterol as well as reduction of sphingomyelin level accompanied by the increase of ceramide content in this model system modify its properties. As it was mentioned above all the foregoing alterations in membrane composition were recognized in various cancer cells supplemented with β-sitosterol.

2. Experimental

2.1. Materials

Egg sphingomyelin (SM), ganglioside GM3 (Milk, Bovine-Ammonium Salt) and C16 ceramide (Cer) were purchased from Avanti Polar Lipids, Inc., while cholesterol (Chol) and β-sitosterol (β-sito) were supplied by Sigma. All the compounds were the products of high purity (≥99%). The respective compounds were weighted out on the analytical balance (accuracy: ±0.01 mg) and then, to prepare the spreading solutions, they were dissolved in chloroform (sterols) or in chloroform/methanol 9:1 v/v mixture (SM, GM3 and Cer). Both chloroform and methanol were purchased from Aldrich, HPLC grade, ≥99.9%. Mixed solutions of desirable compositions were prepared from the respective stock solutions and deposited onto water subphase with the Hamilton microsyringe (accuracy: ±1.0 μL). After spreading the monolayers were left for 10 min to allow for solvent evaporation and then they were compressed with the barrier speed of 20 cm^2/min.

2.2. Methods

The experiments were performed with the NIMA (UK) Langmuir trough (total area = 300 cm^2) placed on an anti-vibration table. Surface pressure was measured with the accuracy of ±0.1 mN/m using Wilhelmy plate made of filter paper (ashless Whatman Chr1) connected to an electrobalance. The subphase temperature (20 °C) was controlled thermostatically to within 0.1 °C by a circulating water system. For the experiments Ultrapure Milli-Q water was used (pH = 6.5 and resistivity of 18 MΩ cm at 20 °C ± 0.1 °C). All the experiments were repeated at least twice to obtained consistent isotherms.

Brewster angle microscopy experiments were performed with UltraBAM instrument (Accurion GmbH, Goettingen, Germany) equipped with a 50 mW laser emitting p-polarized light at a wavelength of 658 nm, a 10× magnification objective, polarizer, analyzer and a CCD camera. The spatial resolution of BAM was 2 μm.

2.3. The investigated mixtures

The starting mixture was Chol/SM/GM3 = 0.3:0.6:0.1 monolayer composed of major lipids found in rafts in breast cancer cells [37]. In the first step of the experiments the composition of these films was modified by partial or total substitution of animal sterol by phytosterol, thus the properties of Chol/β-sito/SM/GM3 = 0.15:0.15:0.6:0.1 and β-sito/SM/GM3 = 0.3:0.6:0.1 monolayers were compared with those of Chol/SM/GM3 = 0.3:0.6:0.1 film. Then, the influence of Cer on sterol/SM/GM3 films was investigated. Therefore, in the initial Chol/SM/GM3 = 0.3:0.6:0.1 mixture and for the comparison in β-sito/SM/GM3 = 0.3:0.6:0.1 film, SM was partially (50%) or totally substituted by Cer. Finally, to reflect all the reported in literature characteristic changes in cancer membrane lipid composition caused by the presence of phytosterol, in the starting Chol/SM/GM3 = 0.3:0.6:0.1 film 50% of cholesterol and 50% of SM were replaced by β-sitosterol and ceramide, respectively, and the properties of Chol/β-sito/SM/Cer/GM3 = 0.15:0.15:0.3:0.3:0.1 mixed film were analyzed.

Additionally, selected binary films composed of the lipids being the components of the studied multicomponent films, namely: sterol/GM3, sterol/Cer, SM/GM3 as well as Cer/GM3, were investigated to facilitate the analysis of the data collected for more complex monolayers.

2.4. Data analysis

The morphology of the studied monolayers was verified based on BAM images taken at various stages of the film compression. Moreover, to get insight into the condensation, miscibility and interactions between the lipids in the respective systems, the parameters calculated from the isotherms were also analyzed. Namely, from the isotherms for the respective monolayers the mean molecular area values (A) at a given surface pressure were determined and analyzed. Then, these values were compared with the areas for ideal mixing of the monolayer components (A^{id}) (Eq. (1)) and the analysis of the excess areas per molecule (A^{Exc}) (Eq. (2)) was done [38].

$$A^{id} = \sum A_i X_i \tag{1}$$

where A_i is the mean area per molecule for the respective one component films, and X_i is the mole fraction of the respective component in the mixed monolayer. A_i calculated from the above equation means a linear combination of the areas of *all* the respective single components and their molar fractions in the mixtures.

$$A^{Exc} = A - A^{id}. \tag{2}$$

Finally, the compression modulus values were calculated from π-A curves (Eq. (3)) [39] to compare the state of the investigated films.

$$C_S^{-1} = -A\ (d\pi/dA) \tag{3}$$

wherein A is the mean area per molecule value at a given surface pressure π.

The foregoing parameters were analyzed at π = 30 mN/m. This value of the surface pressure was chosen because of the correlation between the properties of lipid monolayers and bilayers at higher surface pressure region [40].

3. Results

3.1. The replacement of cholesterol by β-sitosterol in Chol/SM/GM3 film

The surface pressure-area (π-A) isotherms recorded for the initial Chol/SM/GM3 film and for the monolayers of decreased level of cholesterol at constant total sterol content are presented in Fig. 1. The compressional modulus vs. the mean molecular area (C_S^{-1}–A) plots are shown in inset of Fig. 1, while the mean molecular areas (A), the areas for ideal mixing (A^{id}), the excess area per molecule values

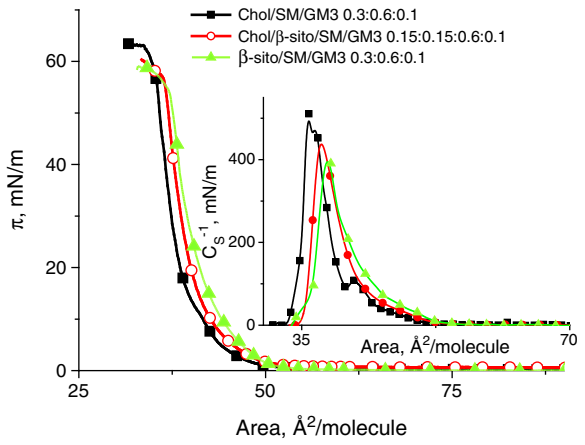

Fig. 1. The surface pressure-area curves for Chol/SM/GM3, Chol/β-sito/SM/GM3 and β-sito/SM/GM3 monolayers. Inset: the compressional modulus vs. the mean molecular area plots for the studied films.

(A^{Exc}) and the compression modulus (C_S^{-1}) values for the studied films at π = 30 mN/m can be found in Table 1.

As it can be observed in Fig. 1 the isotherm for original Chol/SM/GM3 film starts to increase at the area of c.a. 52 Å^2/molecule and collapses at the surface pressure of ca. 62 mN/m. In the course of the isotherm, at π ≈ 12 mN/m, appears a characteristic kink. This kink can be easily identified as a minimum in the compression modulus vs. the mean molecular area plot for this film (inset of Fig. 1). Maximal values of C_S^{-1} below and above this region ($C_S^{-1} \approx 100$ vs. $C_S^{-1} \approx 500$) indicate that with the compression of the monolayer the transition from liquid to condensed state occurs. The excess area per molecule values obtained for this mixed film are negative, which means a non ideal behavior of the system. This also suggests that in the mixed monolayer the molecules interact more favorably than the respective molecules in their one component films.

On the other hand partial (50%) or total replacement of cholesterol in this film by phytosterol causes the shift of the isotherm to larger areas, a decrease of the collapse surface pressure and modifications in the course of the curve. Namely, the slope of the curves changes in respect to that for Chol/SM/GM3 film, which results in lower maximal values of the compression modulus (see inset of Fig. 1 and Table 1) as well as there is a lack of a kink easily noticeable in the curve for Chol/SM/GM3 monolayer. All these findings prove lower condensation and ordering of Chol/β-sito/SM/GM3 and β-sito/SM/GM3 monolayers as compared to Chol/SM/GM3 film. The substitution of cholesterol causes also that A^{Exc} values become less negative as compared to those for initial monolayer. This means weaker deviations from ideality, less favorable interactions between molecules and lower potency of plant sterol as compared to cholesterol as a condensing agent.

To compare morphology of these monolayers, BAM images were taken at different stages of film compression. However, to complete the analysis of the obtained data, BAM pictures for sterols/GM3 and SM/GM3 films, at lipid proportion corresponding to that in Chol/SM/GM3 film, were also examined (Fig. 2). As regards sterol/GM3 mixtures (3:1), it was found that both cholesterol and β-sitosterol cause condensation of ganglioside film. However, the A^{Exc} values calculated from the isotherms for these binary systems (not presented curves) ($A^{Exc} = -2.8$ Å^2/molecule vs. -2.1 Å^2/molecule for cholesterol and β-sitosterol, respectively at π = 30 mN/m) indicate that the effect of cholesterol is stronger than that induced by phytosterol. The differences between these mixed films appear also in BAM images (Fig. 2a and b).

In the pictures for Chol/GM3 (3:1) monolayer at π ≈ 5 mN/m very small circular domains can be observed. Although they are faint, they exist in the texture of the monolayer up to the collapse point. These kind of structures are not observed for β-sito/GM3 monolayer — in this case the images are homogenous and evidence a condensed state of the film. Both these sterols mix favorably also with sphingomyelin [41]. In BAM pictures taken for cholesterol/SM (1:2) films (presented in ref. [42]), at large molecular areas, domains of condensed phase dispersed in gaseous phase were observed. With further compression these domains merged together forming a homogenous condensed film [42].

As regards SM/GM3 = 6:1 film, in BAM images taken for this system (Fig. 2c) at large molecular areas, the coexistence of gas and fluid phase can be observed and then, in the range of π ≈ 3 ÷ 7 mN/m, the film is homogenous (not presented images). Then, that is in the region of phase transition, first irregular condensed domains appear (Fig. 2c). These domains grow both in the number and size with the monolayer compression and above π = 30 mN/m they start to gather together, however, the films remains inhomogeneous. The positive values of A^{Exc} for this mixture ($A^{Exc} = 1.0$ Å^2/molecule at π = 30 mN/m) allow one to conclude that the interactions between lipids are less favorable than those between each molecules in the respective pure monolayer, which together with the film's texture observed in BAM images may indicate immiscibility or segregation within the monolayer. The addition of sterol into SM/GM3 film modifies its morphology (Fig. 3a and b). Considering Chol/SM/GM3 mixture, at large molecular areas, the film is completely homogenous and only a gaseous phase can be detected in the pictures. At the surface pressure of ca. 0.2 mN/m the coexistence of gas and fluid phase can be observed in the images. With further compression the film's texture becomes uniform (up to π < 10 mN/m). Then (that is in the region of phase transition) the domains of a condensed phase are formed. Their number and size increase with film compression up to the collapse point. Partial or total replacement of cholesterol in the foregoing mixture by the plant sterol modifies its morphology in such a way that contrary to Chol/SM/GM3 film the condensed domains are not observed and at higher surface pressures monolayers are completely homogenous (therefore, in Fig. 3 only the images for β-sito/SM/GM3 monolayer are shown). The comparison of the images taken at lower surface pressures for β-sitosterol-containing and cholesterol-containing monolayer indicates a greater condensation of the mixture containing animal sterol.

Table 1
The mean molecular areas (A), the areas for ideal mixing (A^{id}), the excess mean molecular area (A^{Exc}) and the compression modulus (C_S^{-1}) values for the studied films (π = 30 mN/m).

		A, Å^2/molecule (±0.2)	A^{id}, Å^2/molecule (±0.2)	A^{Exc}, Å^2/molecule (±0.4)	C_S^{-1}, mN/m (±10%)
Replacement of Chol by β-sito	Chol/SM/GM3	37.4	41.7	−4.3	410
	Chol/β-sito/SM/GM3	38.7	41.2	−2.5	360
	β-sito/SM/GM3	39.5	40.8	−1.3	260
	Chol/β-sito/SM/Cer/GM3	40.3	40.5	−0.2	440
Replacement of SM by Cer	Chol/SM/Cer/GM3	40.5	40.9	−0.4	550
	Chol/Cer/GM3	43.7	40.2	3.5	610
	β-sito/SM/Cer/GM3	40.5	40.1	0.4	370
	β-sito/Cer/GM3	41.0	39.4	1.6	490

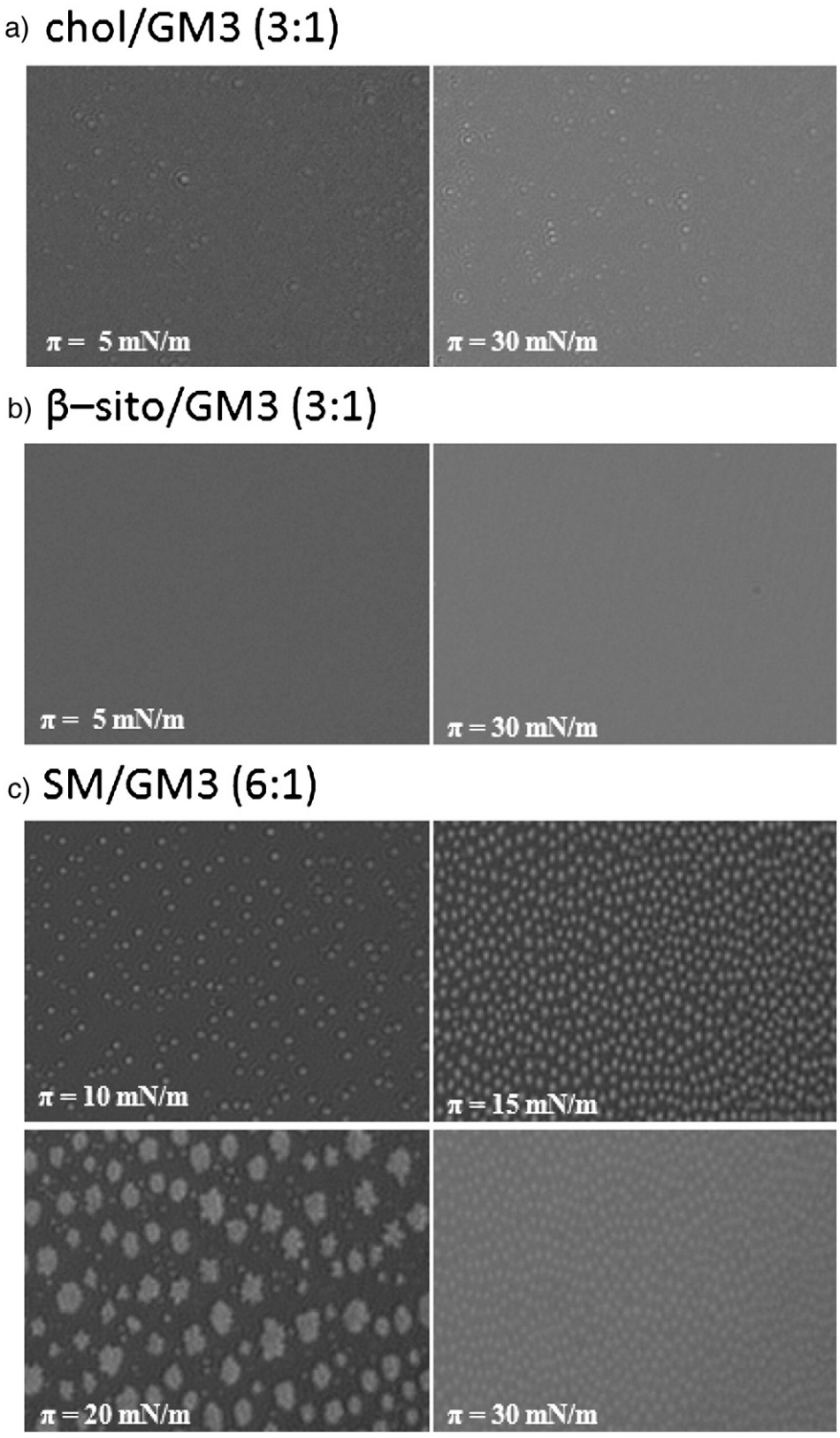

Fig. 2. BAM images taken for binary films composed of sterols and ganglioside GM3 as well as sphingomyelin (SM) and ganglioside GM3.

3.2. The influence of ceramide on sterol/SM/GM3 monolayer

In the next stage of the investigations the effect of ceramide on model membranes was verified in the experiments based on partial or complete replacement of SM by ceramide in Chol/SM/GM3 and β-sito/SM/GM3 monolayers. The π-A curves and the C_S^{-1}–A plots for sterol/SM/GM3 films as well as for sterol/SM/Cer/GM3 and sterol/Cer/GM3 mixtures are shown in Fig. 4a

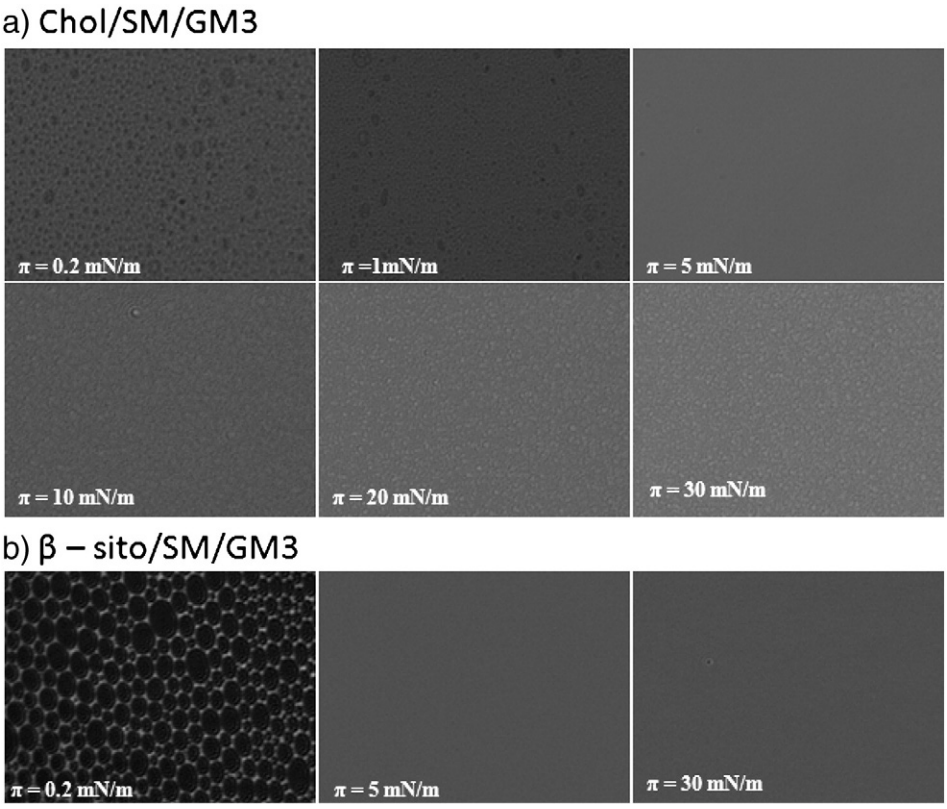

Fig. 3. BAM images taken at different stages of compression of the studied monolayers.

and b. The A^{Exc} as well as C_S^{-1} at 30 mN/m are compiled in Table 1.

As it can be seen in Fig. 4 the addition of ceramide into both sterol/SM/GM3 film causes similar alterations in the recorded curves, namely a decrease of the collapse surface pressure, increase of C_S^{-1} and the shift of the isotherms to larger areas (Fig. 4 and Table 1). Moreover, A^{Exc} values become less negative as compared to those for the respective sterol/SM/GM3 films. At 50% replacement of SM by ceramide the A^{Exc} values at $\pi = 30$ mN/m oscillate around zero, while at total substitution of SM they are positive. This may suggest immiscibility of film components. To resolve this problem, BAM images for these systems and for the respective one-component and binary films were analyzed (Fig. 5).

As regards ceramide (C16) it is known to form monolayers of high condensation [43]. In the images taken at large molecular areas (Fig. 5a) irregular solid structures within gaseous region are clearly noticeable. With film's compression the solid patches merge together and at $\pi \approx 4$ mN/m monolayer is completely homogenous. The incorporation of sterols into ceramide film modifies its texture, however, the observed effect depends on the type of sterol (Fig. 5b and c). At large molecular areas the structures visible in the pictures for β-sito/Cer (Fig. 5c) monolayers are nearly identical with those taken for pure ceramide film (Fig. 5a) however, they differ significantly from those observed for Chol/Cer monolayers (Fig. 5b). In both cases, the compression of the film causes vanishing of gaseous phase and joining the condensed structures. In the case of Chol/Cer monolayer, at $\pi \approx 1$ mN/m the chain-like structures within the condensed phase are formed and the film texture does not change up to the collapse. These kind of structures are not observed for β-sito/Cer film, however, also in this case the monolayer remains inhomogeneous up to the collapse.

It was also reported [44] that Cer molecules interact favorably in monolayers with GM3 [44]. In Fig. 5d BAM images for Cer/GM3 = 6:1 mixtures are shown. As it can be observed, the morphology of this mixture is very similar to that for one component ceramide film. Namely, the platforms of condensed phase coexisting with gaseous region, observed at large molecular areas, systematically join together with the monolayer compression. However, even at high surface pressure region, the film is not uniform since small vague condensed domains are visible in the pictures.

As far as SM/Cer mixtures are concerned, their properties were intensively investigated with various experimental techniques and in a wide range of composition [see e.g. 45,46]. In general, these lipids are of limited miscibility and the formation of ceramide-enriched phase in SM monolayer was evidenced [47,48].

The incorporation of ceramide both into Chol/SM/GM3 and β-sito/SM/GM3 monolayer drastically changes the morphology of these films. Comparing the images for Chol/SM/GM3 in Fig. 3a with the pictures shown in Fig. 6a and b it can be concluded that the replacement of SM by Cer induces phase separation in the monolayer. At lower surface pressures the condensed domains within less condensed phase are visible, however, with the film compression the formation of a number of bright domains of higher condensation within inhomogeneous monolayer can be observed (Fig. 6a). In the pictures taken for Chol/Cer/GM3 film (Fig. 6b) irregular structures of condensed phase together with a number of bright domains forming chain-like structures and being of higher condensation are visible within the whole range of the surface pressures.

The morphology of β-sitosterol-containing mixtures was found to be different. Namely, in the images for β-sito/SM/Cer/GM3 film (Fig. 6c), at $\pi \geq 2$ mN/m, a number of domains differing in condensation can be observed. Total replacement of SM by Cer (Fig. 6d) causes that, at very low surface pressure, characteristic patches are formed, which are nonuniform as regards their condensation degree. These patches merge together with film's compression and form more uniform and highly condensed phase within homogenous and less condensed matrix.

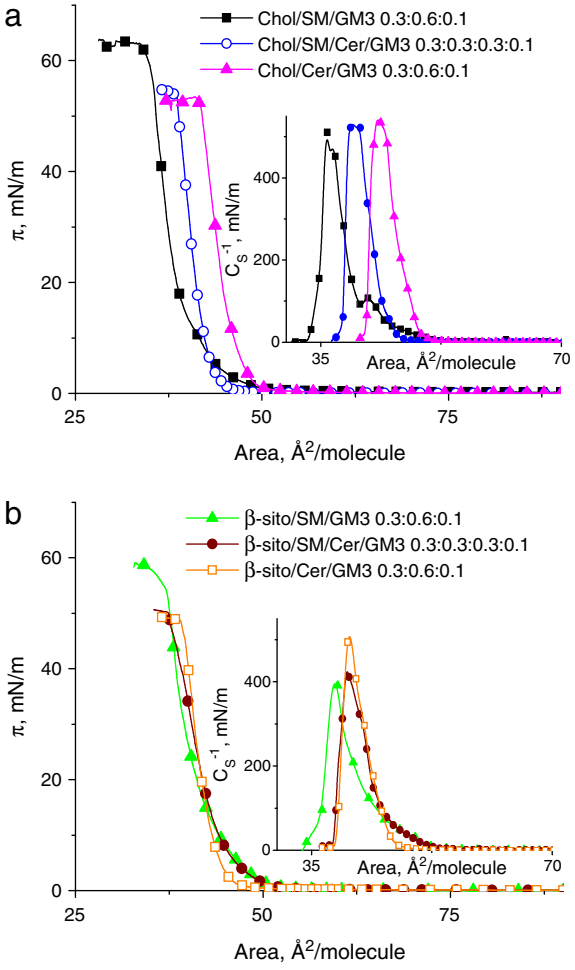

Fig. 4. The surface pressure-area curves for sterol/SM/GM3, sterol/SM/Cer/GM3 and sterol/Cer/GM3 monolayers, Inset: the compression modulus vs. the mean molecular area plots.

3.3. The properties of Chol/β-sito/SM/Cer/GM3 films

In a final step of the investigations the composition of Chol/SM/GM3 model membrane was altered by the replacement of 50% of cholesterol by β-sitosterol and 50% of SM by ceramide.

As results from Fig. 7 and Table 1, the foregoing modifications in the composition of Chol/SM/GM3 mixture decrease packing of molecules in the film, which reflects in larger mean molecular areas, causes destabilization of the monolayer, which is manifested in lower collapse surface pressure, makes the interactions less favorable as compared to those in Chol/SM/GM3 monolayers, which is reflected in much less negative A^{Exc} values. On the other hand, the maximal value of C_S^{-1} is very similar for both films, which indicates similar rigidity of these systems. In the isotherm for Chol/β-sito/SM/Cer/GM3 film, similarly to Chol/SM/GM3 curve, the kink is visible, however, it appears at higher surface pressure (15 mN/m) and separates the region of higher condensation. The maximal values of compression modulus below and above minimum in C_S^{-1} vs. A plots ($C_S^{-1} \approx 260$ vs. $C_S^{-1} \approx 520$) indicate that the state of monolayer changes from liquid condensed to solid condensed [39] or rather from tilted condensed to untitled condensed [49]. Similar phase transition was detected e.g. in DPPG and DPPE monolayers [50].

Both the A^{Exc} values, which are very close to zero as well as BAM pictures (Fig. 8) evidence the occurrence of phase separation in Chol/β-sito/SM/Cer/GM3 film. As it can be seen (Fig. 8), at low surface pressures, within the monolayer coexist gaseous, fluid and large platforms of condensed phase. Moreover, within the condensed regions containing gaseous holes, the small domains of greater condensation are also observed. The compression of the monolayer causes vanishing of gaseous regions, however, in all range of the surface pressure, in the images the coexistence of various phases of different degree of condensation and homogeneity can be observed. Additionally, even at low surface pressure, small bright domains of 3D phase appear.

4. Discussion

Supplementation of cancer cells with β-sitosterol entails strong alterations in the composition of membranes, that is a decrease of cholesterol level and its substitution by plant sterol as well as a decrease of sphingomyelin concentration and increase of ceramide content. In this work it was verified how these modifications change the organization of model Chol/SM/GM3 system.

Cholesterol and β-sitosterol are known to be of different condensing and ordering potency and interactions with other membrane lipids. The studies on binary and multicomponent model membranes showed that phytosterol is less effective in modulation of the properties of the systems composed of major membrane phospholipids (phosphatidylcholines, sphingomyelin [e.g. 41,51,52]). The results obtained in this work allow one to expand these studies by comparing the effect of these sterols on ganglioside and ceramide monolayers. As evidenced fluorescence microscopy experiments, at 20% of cholesterol in sterol/GM3 mixture, the domains, probably enriched in cholesterol, are formed in the system [53]. Such domains appear also in BAM images presented herein for Chol/GM3 films, however, they are undetectable in the pictures for β-sito/GM3 monolayer. This fact, together with higher (less negative) values of the excess area per molecule obtained for β-sito/GM3 monolayer as compared to Chol/GM3 film, confirms weaker condensing effect of plant sterol vs. animal sterol on GM3 monolayers. Thus, cholesterol is able to pack in GM3 film more beneficially than β-sitosterol. This is due to molecular structure of cholesterol, which ensures better fit and more favorable interactions between hydrophobic part of molecules as compared to plant sterol having larger diameter caused by the presence of a branched side chain [54]. These differences in the properties of cholesterol vs. phytosterol allow one to understand the results obtained herein for multicomponent films. Namely, it was found that the replacement of cholesterol by β-sitosterol in Chol/SM/GM3 film weakens the interactions between molecules, decreases film's condensation and stability. Similar effects were found also in previous studies [55,56]. However, the analysis of BAM pictures proves also, that the substitution of cholesterol in Chol/SM/GM3 by β-sitosterol inhibits the formation of condensed domains in the system.

The results obtained herein evidenced also differences in the effect of both sterols on ceramide monolayer. Comprehensive investigations made by Scheffer et al. [57] proved, that the organization of Chol/Cer film is determined by the proportion of lipids in the system. Namely, in the mixtures dominated by cholesterol (up to 33 mol% of Cer) the mixed crystalline phase is formed within the film. In the range of Chol:Cer proportion between 50:50 ÷ 30:70 the coexistence of mixed crystalline phase and ceramide crystalline phase was found, while at higher level of Cer only crystalline phase of ceramide was detected [57]. Based on the foregoing findings, the chain-like structures observed in a wide range of the surface pressure in BAM pictures taken for Chol/Cer = 1:2, can be identified as a ceramide-rich phase coexisting with mixed phase of both film components. However, the structures observed in BAM images for β-sito/Cer = 1:2 film were found to be of completely different organization. Interestingly, they were similar to the vague structures identified in AFM pictures for Chol/Cer film of very low (5%) cholesterol proportion [58]. Although the level of sterol in the studied herein binary β-sito/Cer film is higher than 5%, it should be kept in mind that the plant sterol is of different structure and properties than cholesterol and its influence on lipids

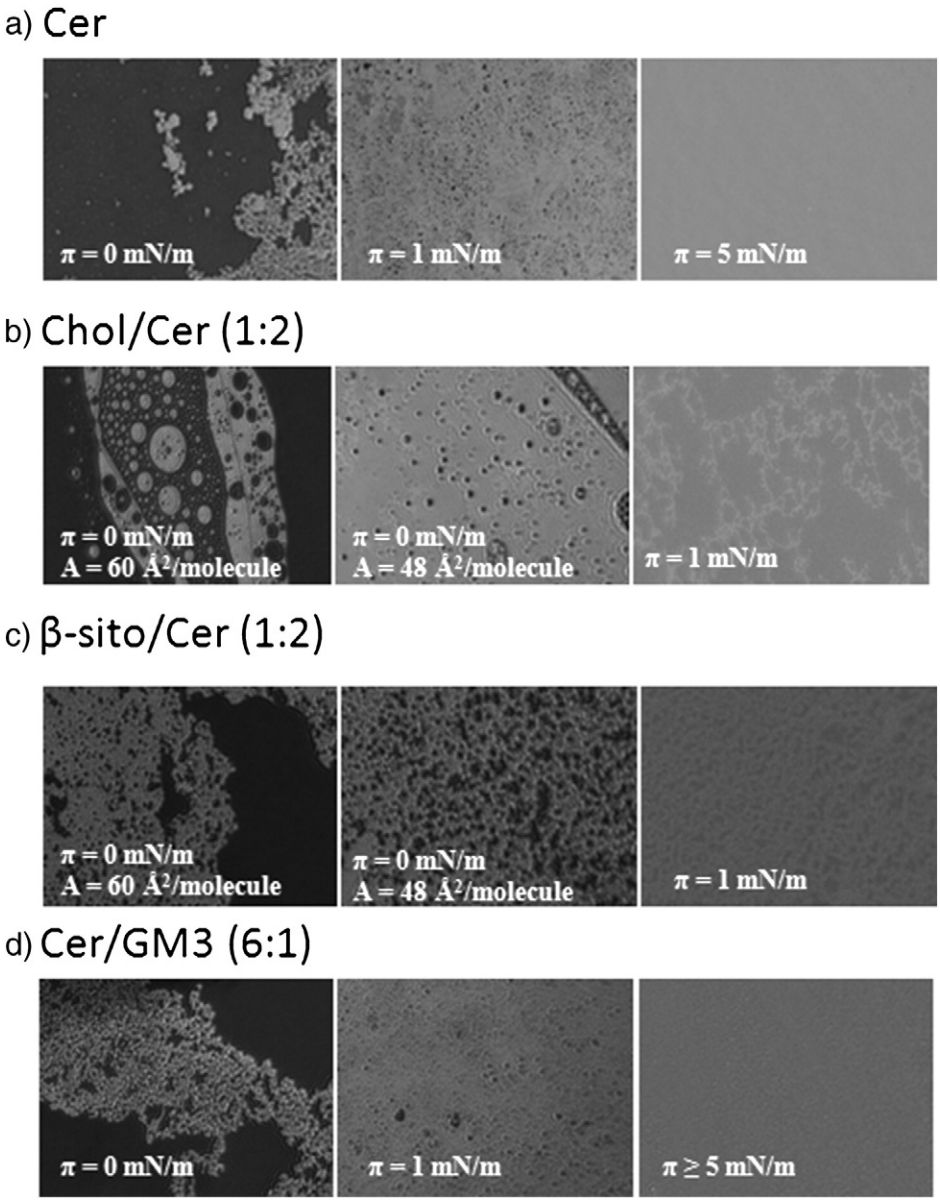

Fig. 5. BAM images for ceramide, sterol/ceramide and ceramide/GM3 monolayers.

films is weaker than cholesterol at the same content in the mixed system. Therefore, it can be assumed that the morphology of β-sito/Cer monolayer at a given concentration of phytosterol may be similar to that for Chol/Cer film, however, at lower cholesterol content. Thus, it can be suggested that within the studied herein β-sito/Cer film, the ceramide crystalline phase tends to be formed, similarly to Chol/Cer monolayer of lower cholesterol content [57].

The collected data evidenced that the addition of ceramide into sterol/SM/GM3 monolayer causes drastic modifications in the morphology of the films, however, the observed variations strongly depend on the kind of sterol in the system. The analysis of BAM images and A^{Exc} values calculated from the isotherms leads to the conclusion that incorporation of ceramide into both sterol/SM/GM3 films causes that the lipids tend to phase separation. In cholesterol-containing monolayers crystalline domains are formed during film compression, both after partial or total replacement of SM by ceramide. At partial substitution of SM in phytosterol-containing film, in the whole range of the surface pressure, the domains of different condensation exist. Total replacement of SM by Cer molecules results in the formation of larger patches, which join together and form highly condensed platforms within less condensed matrix. Thus, the incorporation of ceramide into both these mixtures provokes phase separation in the systems, however, the morphology of the films is completely different and determined by the kind of sterol in the monolayer. Although ceramide strongly modifies the organization of the studied herein films, the alterations in the morphology of the initial Chol/SM/GM3 monolayer were found to be even greater after simultaneous partial substitution of cholesterol by plant sterol and SM by ceramide. Namely, the compression of Chol/β-sito/SM/Cer/GM3 monolayer in the range of the surface pressure of 0–20 mN/m allows one to observe in BAM images large diversity of phases formed within the film, including the coexistence of homogenous LC phase, the number of the condensed domains as well as crystalline domains increasing with film compression.

The phenomenon of the promotion of heterogeneity in lipid mixtures by the incorporation of ceramide is due to the ability of this lipid to self-associate and form ordered domains. As it was evidenced in the studies on various lipid systems, long chained (N-acyl chain longer than C12) ceramides added into lipid environment tend to segregate and in general generate gel-like ceramide-enriched phase

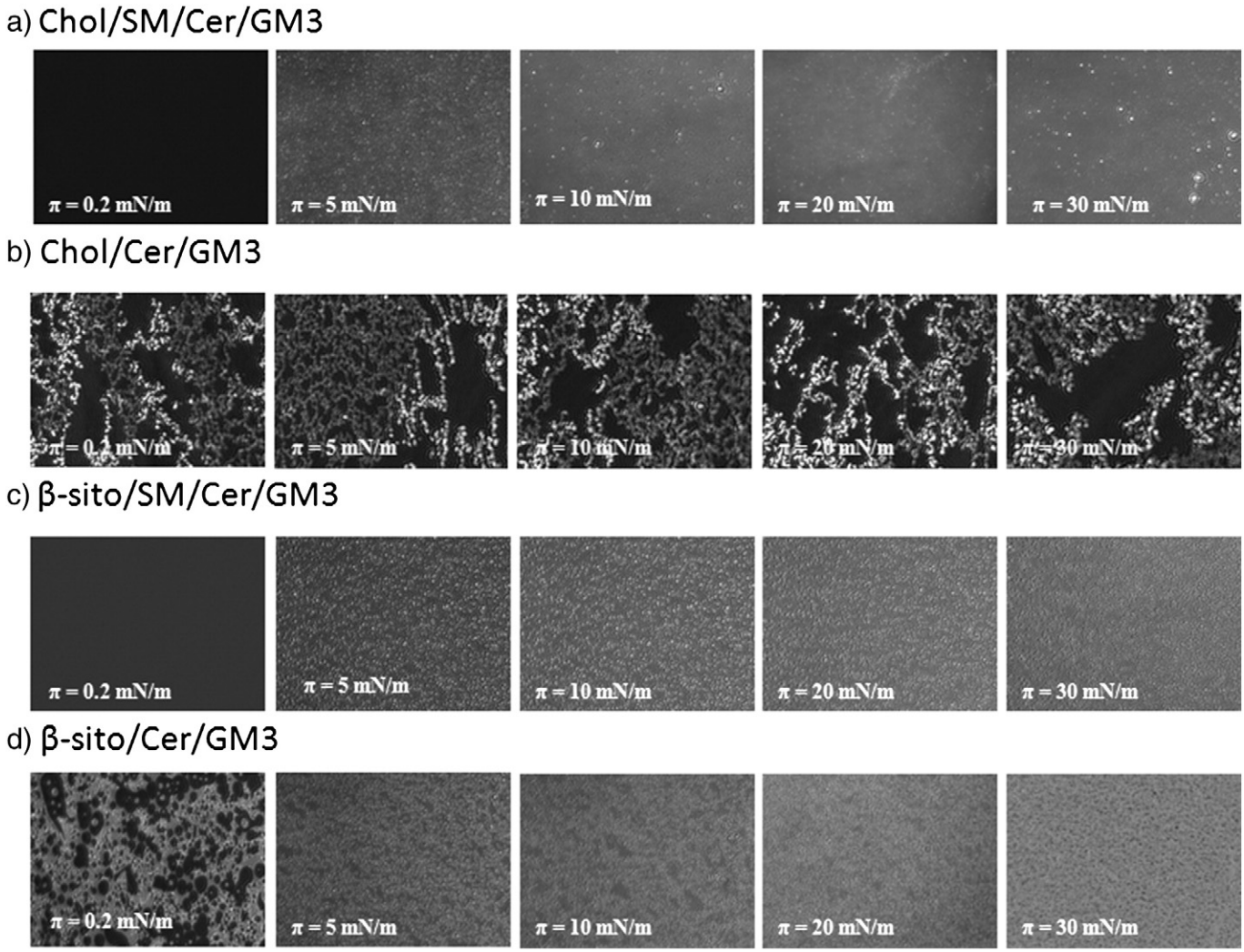

Fig. 6. BAM images taken for the studied monolayers.

[45–47,57,59,60]. Based on the experiments on bovine brain SM and CerIII it was postulated, that at lower surface pressures the molecules are partially miscible, while at higher surface pressures partial immiscibility and formation of ceramide-enriched phase in SM monolayer were evidenced [47]. However, the behavior of ceramide in lipids system is more complicated and the formed structures are of more complex nature and organization. For example, it was suggested [45] that LC ceramide-enriched phase formed in SM/Cer systems ($X_{Cer} > 0.4$) is a mixture of at least two different LC phases. Similarly, the addition of Cer into DOPC/SM/Chol mixtures was found to promote heterogeneity within the system and lead to the formation of heterogeneous, irregular domains, in which ceramide is concentrated in subdomains [46]. Moreover, AFM results for the foregoing system indicated very small differences between ceramide-enriched and ceramide-poor domains [46].

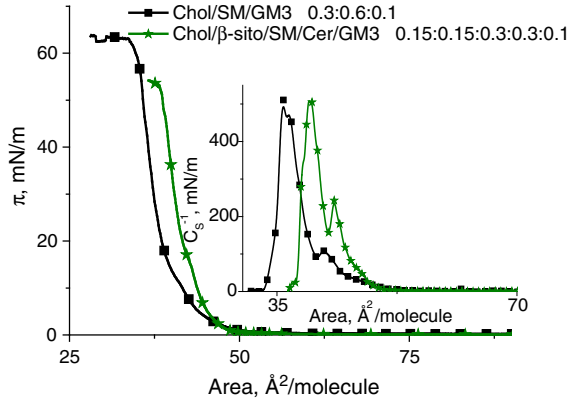

Fig. 7. The surface pressure-area curves and the compression modulus vs. the mean molecular area plots for Chol/SM/GM3 and Chol/β-sito/SM/Cer/GM3 films.

The investigations on the effect of ceramide on raft model system proved that the alterations in lateral organization of lipids in the studied mixtures caused by Cer depend on the fraction of *lo* phase and the size of model rafts [61]. It was also found that ceramide competes with cholesterol in the interactions with sphingomyelin and displaces them from ordered domains [36,61,62]. All of this proves that the effect of ceramide on lipids is highly complex and, depending on exact lipid composition, ceramide structure and its content in the system, various phases, differing in the composition and morphological features, may be formed. Therefore, based on the results obtained herein it is impossible to certainly indicate the nature of the structures observed in the images taken for the studied multicomponent monolayer. However, the collected results allow one to conclude that the incorporation of β-sitosterol and ceramide into model membranes instead of cholesterol and sphingomyelin, respectively, significantly modifies the organization of lipids in the monolayer and film's

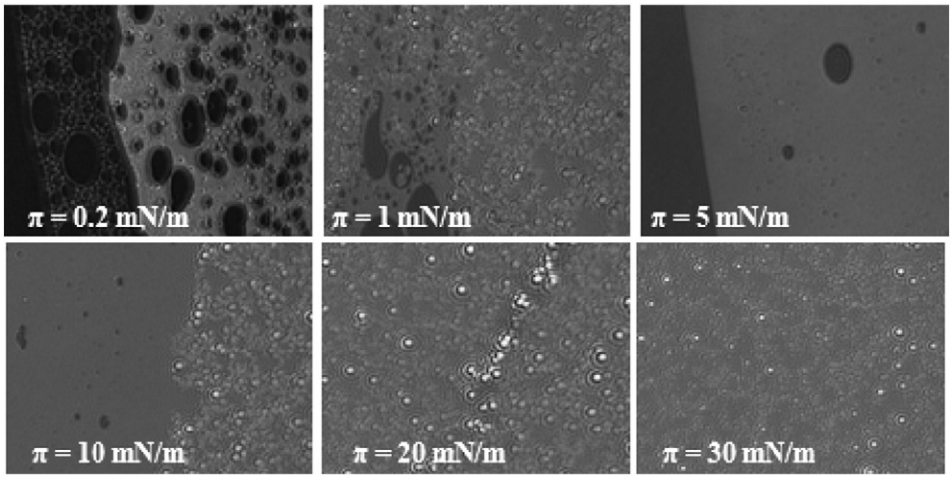

Fig. 8. BAM images taken for Chol/β-sito/SM/Cer/GM3 = 0.15:0.15:0.3:0.3:0.1.

morphology. It can be thus suggested that the occurrence of this kind of modifications in natural systems may strongly disturb physicochemical properties and morphology of membrane and may be critical for cell viability. Although inhibitory effect of this plant sterol on the growth of various cancer cells was found to be connected with serious alterations in signaling pathways and apoptosis, the segregation of lipids within membranes and rafts may be a decisive step in foregoing activity of β-sitosterol.

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